# REQUIREMENTS FOR CELL - FREE CYANIDE OXIDATION BY *Pseudomonas fluorescens* NCIMB 11764.

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The involvement of cyanide oxygenase in the metabolism of pyruvate and α-ketoglutarate-cyanohydrin was investigated and shown to occur indirectly by the consumption of free cyanide arising from the cyanohydrins via chemical dissociation. Thus, free cyanide remains the substrate, for which the enzyme displays a remarkably high affinity ($Km_{app}$,4 μM). A model for cyanide utilization is therefore envisioned in which the substrate is initially detoxified by complexation to an appropriate ligand followed by enzymatic oxidation of cyanide arising at sublethal levels via chemical dissociation. Putative cyanide oxygenase in cell extracts consumed both oxygen and NADH in equimolar proportions during cyanide conversion to $CO_2$ and $NH_3$ and existed separately from an unknown heat-stable species responsible for the nonenzymatic cyanide-catalyzed consumption of oxygen. Evidence of cyanide inhibition and nonlinear kinetics between enzyme activity and protein concentration point to a complex mechanism of enzymatic substrate conversion.

ACKNOWLEDGEMENTS

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TABLES OF CONTENTS

LIST OF ILLUSTRATIONS

# CHAPTER I

# INTRODUCTION

## 1. Occurrence of cyanide in nature

The production of cyanide (cyanogenesis) as a natural product represents an important source of this toxic compound in the environment. Over 2000 species of plants are cyanogenic as are various fungi and certain bacteria (Conn, 1980; Castric, 1981; Poulton, 1988; Reed, 1988; Rumack, 1983). While the role of biological cyanogenesis is not entirely clear it is generally believed to play a role in defense against biological competitors. In addition to biological sources, cyanide can also arise anthropogenically. Large quantities employed in various industrial processes such as metal plating, steel tempering and mining (Agency for Toxic Substances and Disease Registry, 1993; Homan, 1988; Towill et al., 1978) and represent important sources of potential cyanide pollution in the environment. Since cyanide is toxic at concentrations as low as $10^{-4}$ M (2.6 ppm) its removal from the environment takes on special significance (Dixon and Webb, 1964). Industrial means of cyanide destruction generally involve chemical treatment, but these can be expensive and are not always risk free. Biological means of cyanide treatment which are both cost effective and risk free, have therefore been adopted, particularly in the mining industry where cyanide can be mineralized to harmless end products (Mudder and Whitlock, 1984).

2. Physicochemical properties and toxicity of cyanide.

Hydrogen cyanide (free cyanide) is a weak acid (pKa 9.3) that boils at room temperature (25$^{o}$C)(Fuller, 1984). This means that at physiological pH (7.0) cyanide exists predominantly (>99%) in the protonated form with an equilibrium between dissolved and gaseous species. Being a strong nucleophile, it reacts readily with metals, particularly those of the transition series to form metal complexes (Sharp, 1976). Its toxicity is attributed mainly to the inhibition of metalloenzymes, most notably, cytochrome oxidase, the terminal component of respiratory chains (Solomonson, 1981). This enzyme is a multienzyme complex containing two heme *a* components and two $Cu^{2+}$ ion oxidation reduction centers of which the $Cu_B^{2+}$ site is involved in initial cyanide binding. The fully oxidized form has a two fold higher affinity for cyanide than the reduced form, but binding is relatively slow. Apart from cytochrome oxidase, other metalloenzymes including those containing molybdenum (nitrate reductase, xanthine oxidase, sulfite oxidase) copper (Cu superoxide dismutase) and zinc (Zn superoxide dismutase, alkaline phosphatase, and carbonic anhydrase) also display marked cyanide sensitivity. In all cases sensitivity is attributed to complexation of the metal prosthetic group. However, various non-metalloenzymes also demonstrate cyanide sensitivity such as ribulose-2,3-bisphosphate carboxylase in which case cyanide appears to react with ribulose diphosphate forming a cyanohydrin derivative that slows enzyme activity .

3. Biological tolerance to cyanide.

In spite of its toxicity, some organisms show a remarkable tolerance to cyanide. One mechanism found universally in microorganisms and plants for increasing tolerance to

cyanide involves the elaboration of cyanide-resistant cytochrome oxidases ( Bonner et al, 1986; Cunningham and Williams, 1995; Huq and Palmer, 1978; Kay and Palmer, 1985; Rhoads et al, 1998). In response to cyanide, for example, *Pseudomonas aeruginosa* and *Bacillus cereus* elaborate cytochrome oxidases capable of tolerating inhibitor concentrations as high as 1 mM (Knowles and Bunch, 1986).

A second important means of acquiring cyanide tolerance is to produce detoxifying enzymes. Enzymes capable of chemically transforming cyanide and thus neutralizing its toxicity have been described in mammals, higher plants and microorganisms. The main enzyme responsible for cyanide detoxification in mammals is rhodanese (thiosulfate cyanide sulfurtransferase)(E. C. 2.8.1.1). This enzyme catalyses a reaction between cyanide and thiosulfate forming less toxic thiocyanate (equation 1).

$$S_2O^{-}_3{}^2 + HCN \xrightarrow{\text{Rhodanese}} HSCN + SO^{-}_3{}^2 \qquad \text{Equation 1}$$

In plants the principal enzyme believed to be responsible for cyanide detoxification is β-cyanoalanine synthase (E. C. 4.4.1.9) (Castric P.A. 1981). This enzyme catalyzes the condensation of cyanide with a three carbon unit compound such as cysteine (or acetyl-L-serine)(equation 2); this reaction is also catalyzed by cysteine synthase (E. C. 4.2.99.8).

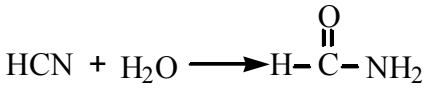
Equation 2

$$HSCH_2{-}\underset{\displaystyle NH_2}{\overset{\displaystyle COOH}{\underset{|}{\overset{|}{C}}}}{-}H + HCN \xrightarrow{\beta\text{- cyanoalanine synthase}} \underset{\beta\text{-cyanoalanine}}{COOH{-}\overset{\displaystyle H_2N}{\overset{|}{C}}H{-}CH_2{-}C\equiv N} + H_2S$$

β-cyanoalanine synthetase has also been detected in *E. coli* ( Dunnill and Fowden, 1965),

*Bacillus megaterium* (Castric and Strobel, 1969) and *Chromobacterium violaceum* (Brysk et al, 1969).

An additional enzyme believed to be involved in cyanide tolerance is cyanide hydratase (E.C. 4.2.1.66). This enzyme has thus far been described primarily in phytopathogenic fungi (*Stemphylium loti, Gloecercospora sorghi* ) and is responsible for the conversion of cyanide to less toxic formamide (Fry and Millar, 1972; Nazly et al, 1983; Wang et al, 1992; Cluness et al, 1993) (equation 3).

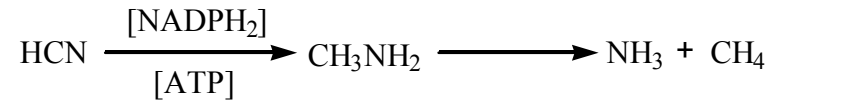


Equation 3

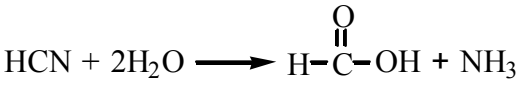
4. Microbial utilization of cyanide as a growth substrate.

A number of reports describing the growth of microorganisms on cyanide have appeared over the years. Early descriptions of bacteria able to grow on cyanide as a carbon and nitrogen source have never been substantiated and there are no reports of it being able to serve as both a carbon and energy source (Knowles, 1976; Knowles and Bunch, 1986; Kunz, pers. comm.). This is believed to be due to the fact that at the concentrations needed for this purpose it is too toxic. However, a number of reports of microorganisms able to grow on cyanide as the sole nitrogen source have appeared (Furuki et al., 1972; Harris and Knowles, 1983a; Silva-Avalos et al., 1990). As far as is known, this ability depends on the synthesis of enzymes capable of converting cyanide to ammonia, which can then be assimilated by most organisms. Three separate enzymes capable of converting cyanide to ammonia have been described. These include:

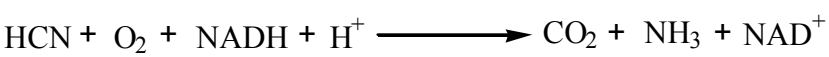
(i) nitrogenase (equation 4) (Hardy and Knight, 1967), (ii) cyanide nitrilase (also referred to as cyanide dihydratase or cyanidase) (equation 5) (Meyers et al., 1993; Ingvorsen et al. 1991; Watanabe et al., 1998) and (iii) cyanide oxygenase (equation 6) (Harris and Knowles, 1983; Knowles, 1988; Kunz et al., 1994; Wang et al., 1996).




Equation 4




Equation 5




Equation 6

While the first two of these are known to produce ammonia as a reaction product there is no evidence that they function physiologically in cyanide utilization. In contrast, strong evidence that CNO is needed for physiological growth comes from work demonstrating that mutants unable to make the enzyme cannot grow. CNO has thus far been described in one organism only, namely, *Pseudomonas fluorescens* NCIMB 11764 (Harris and Knowles, 1983b; Knowles and Bunch, 1986; Kunz et al., 1994). Because of its unique properties, our laboratory has been actively engaged in characterizing its role in the assimilation of cyanide as a growth substrate.

5. Mechanism of cyanide assimilation by *P. fluorescens* NCIMB 11764.

Harris and Knowles (1983a) first proposed that cyanide degradation in *P. fluorescens* NCIMB 11764 occurred oxygenatively since both oxygen and reduced pyridine nucleotide (NADH) were required and utilized in an apparent 1:1 stoichiometry. These initial studies were later confirmed by Kunz and coworkers (1994) who showed

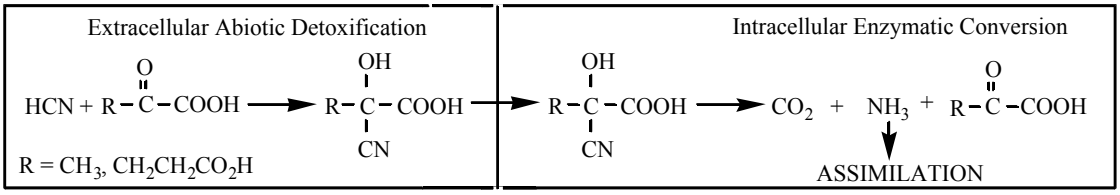
that mutants having lost the ability to oxidize cyanide could no longer grow on cyanide but retained the ability to grow on low concentrations of ammonia. The enzyme responsible for initiating cyanide degradative attack was designated cyanide oxygenase (CNO), however, the nature of the reaction catalyzed by the enzyme, and whether additional enzymes were also required was not established. Subsequent investigations by Wang et al., (1996) showed that CNO functions as a monoxygenase since a single atom of isotopically labeled oxygen-18 was incorporated during conversion. Since the second atom of oxygen recovered in the reaction product $CO_2$ was shown to be derived from water, a reaction mechanism in which cyanide undergoes initial monoxygenative attack followed by hydrolysis of an unknown oxygenated intermediate (X-OH) was proposed (equations 7 and 8). The nature of this intermediate remains unknown, but it is not believed to be cyanate (HOCN) as earlier suggested by Harris and Knowles (1983b). This conclusion is based on the findings of O. Nagappan (Nagappan, 1992) who demonstrated

$$HCN + O_2 + NADH + H^+ \longrightarrow [X\text{-}OH] + NAD^+ + H_2O \qquad \text{Equation 7}$$

$$[X\text{-}OH] + H_2O \longrightarrow CO_2 + NH_3 \qquad \text{Equation 8}$$

that while *P. fluorescens* NCIMB 11764 is capable of growth on cyanate, cyanate-defective mutants remain able to grow on cyanide. In addition, cells grown on cyanide are not induced for cyanase (E.C.3.5.5.3), the principal enzyme responsible for cyanate degradation in this organism. Conversely, cells grown on cyanate are not induced for CNO.

In addition to the induction of CNO, studies by Chen (Chen and Kunz, 1997; Chen, 1998; Kunz et al., 1998) demonstrated that the excretion of keto acids was also essential for growth on cyanide. These studies showed that both pyruvate and α-ketoglutarate react chemically with cyanide, neutralizing its toxicity. The products formed were identified as the corresponding α-hydroxynitriles (cyanohydrins), and a mechanism wherein cyanide is first detoxified outside of cells by chemical conversion to the cyanohydrins prior to being metabolized internally as the cyanohydrin derivatives was proposed (scheme 1)(Kunz et al., 1998). Results demonstrating that pyruvate cyanohydrin (Pyr-CN), radiolabelled with $^{14}C$ in the cyano group, was converted to radiolabelled $^{14}CO_2$ by cell extracts were further consistent with the proposed mechanism.




**Scheme 1. Proposed mechanism for cyanide detoxification and assimilation in *P. fluorescens* NCIMB 11764.**

Moreover, findings which showed that the cell-free conversion of Pyr-CN (and α–ketoglutarate cyanohydrin [Kg-CN]) required both NADH and oxygen, and did not occur in a CNO-defective mutant (JL102) suggested strongly that the further metabolism of the cyanohydrins was CNO-dependent. However, whether the cyanohydrins served directly as substrates for the enzyme or were metabolized by some other mechanism was not

established. The mechanism of cyanohydrin bioconversion by *P. fluorescens* NCIMB 11764 was therefore investigated. These studies show that in fact, CNO does not attack the cyanohydrins directly, but instead consumes free cyanide, which arises at low concentrations from the cyanohydrins by chemical dissociation (Part I). Having discovered this, further studies were undertaken to more fully characterize the requirements and conditions for cyanide oxidation at the cell-free level. These studies now show that cyanide can be oxidized at concentrations far lower than that previously recognized (as low as $\leq 1$ μM), which has important implications for understanding the physiological basis of cyanide tolerance and utilization. Further studies show that enzymatic cyanide oxidation is complex with cyanide serving not only as an substrate but also as an inhibitor of a novel enzyme system which likely is multicomponent in nature. These studies pave the way for further investigation of the molecular basis of cyanide oxidation at the protein level.

# CHAPTER II

# MATERIALS AND METHODS

## 1. Cultivation conditions and methods of enzyme induction.

*P. fluorescens* NCIMB 11764 was routinely subcultured at 4-6 week intervals on L-agar plates (Lennox media [Lennox, 1955]). For the induction of cyanide oxidation activity in cell extracts, colonies were taken from a 36 hour-old plate and transferred to 100 ml minimal glucose-ammonia (GA) medium the composition of which has previously been described (Wang, 1995; Chen and Kunz, 1997). This culture was incubated for 48 h at 30$^{o}$C on gyratory shaker (220 rpm) and the entire culture (10% inoculum) transferred to 1 liter of minimal GA medium, which was incubated for 24 h before adding 0.1 mM KCN to induce cyanide oxidation activity. Cells were harvested 3-5 h thereafter by centrifugation, washed twice with $Na_2HPO_4$-$KH_2PO_4$ (pH 7.0) buffer (Sorenson's Na-K phosphate buffer) and stored at –70$^{o}$C until use.

## 2. Preparation of cell extracts

Washed cells were resuspended in chilled 50 mM Na-K phosphate buffer (pH 7) containing 50 μg/ml DNAse, in the ratio of 2 ml of buffer per gram of cells. The cells were then broken in a chilled French press at 20,000 psi and the resultant extract held at room temperature for 5 minutes to allow time for DNA digestion before centrifugation at 30,000 g for 30 minutes at 5$^{o}$C. The pellet was discarded and the supernatant subjected to

further ultracentrifugation at 150,000 g for 90 minutes. Cyanide oxidation activity was recovered in the high speed supernatant (referred to throughout as cell extract), which was stored at -70$^{o}$C for up to 9 months with no significant loss in activity.

3. Enzyme assays

a. Cyanohydrin bioconversion. The transformation of either Pyr-CN or Kg-CN was determined by measuring the time-dependent disappearance of substrate colorimetrically or by HPLC (Analytical Methods). Reactions mixtures (0.25 ml) contained, 2 mM cyanohydrin, 4 mM NADH and cell extract (1-4 mg) in 50 mM Na-K phosphate buffer (pH 7.0). As the cyanohydrins are unstable at pH 7.0, acidic solutions of each (prepared as described in Chemicals), were pH adjusted to 7.0 (by adding 1 part 5 M Na-K phosphate buffer [pH 7.0] to 10 parts of 50 mM cyanohydrin [pH 2.0)]) immediately before addition to reaction mixtures. Reactions were initiated by the addition of cyanohydrin into sealed vials (2 ml, Rainin HPLC vials) and the remaining cyanohydrin concentration determined by removing samples at periodic intervals.

b. Cyanide oxidation activity. Cyanide oxidation activity was assayed by any one of the several ways. The standard assay involved measuring the time-dependent consumption of cyanide from reaction mixtures similar to that described above except that KCN instead of cyanohydrin was provided as the substrate. Alternatively, activity was assayed by measuring $O_2$ consumption with an oxygen electrode or by following NADH oxidation spectrophotometrically at 340 nm. Since both are required for cyanide oxidation, and particularly by CNO, either assay serves as a convenient method for measuring the activity of this enzyme directly.

Oxygen consumption was measured with a Clark-type $O_2$ electrode (Rank Brothers) calibrated by measuring $O_2$-consumption associated with catechol cleavage by catechol-2,3-dioxygenase (E.C.1.13.11.2), (which has an established reaction stoichiometry of 1:1). Cyanide-dependent $O_2$ consumption was measured in reaction mixtures (0.5-1 ml) containing 50 mM Na-K phosphate buffer (pH 7.0), 1 mM NADH, variable amounts of KCN, and cell extract (1-2 mg protein). Cyanide-dependent NADH oxidation was measured similarly except that reaction mixtures were supplied 0.4 mM NADH in a reaction volume of 0.5 ml. The stimulation in rate of $O_2$ or NADH consumption above that observed in its absence was used to quantify cyanide oxidation activity.

Enzymatic activity was also determined by measuring radiolabelled $^{14}CO_2$ production from $K^{14}CN$. For this purpose, reactions were conducted in a reaction vessel containing a 2 ml HPLC vial placed inside a sealed 15 ml crimp-seal bottle (Pierce). Reactions were terminated by the addition of 25 µl 6N $H_2SO_4$ to the inner vial after which 0.4 ml 4N NaOH was added to the outer reaction compartment to trap volatile radioactive compounds ($Na^{14}CO_3$, $Na^{14}CN$). The contents of both the inner HPLC vial and outer reaction compartment were then fractionated with $BaCl_2$ as previously described (Wang et al, 1996), and radioactivity counted in a liquid scintillation counter (Beckman LS 7000).

4. Analytical methods.

a. Determination of cyanide. Cyanide was determined by the colorimetric method of Lambert et al., (1975) as previously described in our laboratory (Wang, 1995; Chen,

1998; Kunz et al., 1998). Samples 10-50 µl were added to 1.14-1.10 ml water containing 50 µl of N-chlorosuccinimide/ succinimide (0.1 and 1% w/v, respectively) to which was added 50 µl of barbituric acid-pyridine reagent (6 % in 30% aqueous pyridine) and the $A_{580}$ was determined after 15 minutes.

b. Determination of cyanohydrins. Pyr-CN and Kg-CN were determined colorimetrically by adding 20 µl of sample to 80 µl of picric acid reagent (0.25% [w/v]) in 1.25 % $Na_2CO_3$ (Fry and Millar, 1972). The mixture was heated for 8 minutes at 95$^{o}$C and the $A_{480}$ determined after dilution to 1 ml. The cyanohydrin concentration was determined from a standard curve (Fig. 1). HPLC analysis of the cyanohydrins was conduced by ion-exclusion chromatography on an Aminex Ion Exclusion HPX-87H column with 0.015 N $H_2SO_4$ in 0.00034 M EDTA as the mobile phase. Retention times for pyruvate cyanohydrin and α–ketoglutarate cyanohydrin were 8.3 min and 7.4 min respectively.

5. Instrumentation.

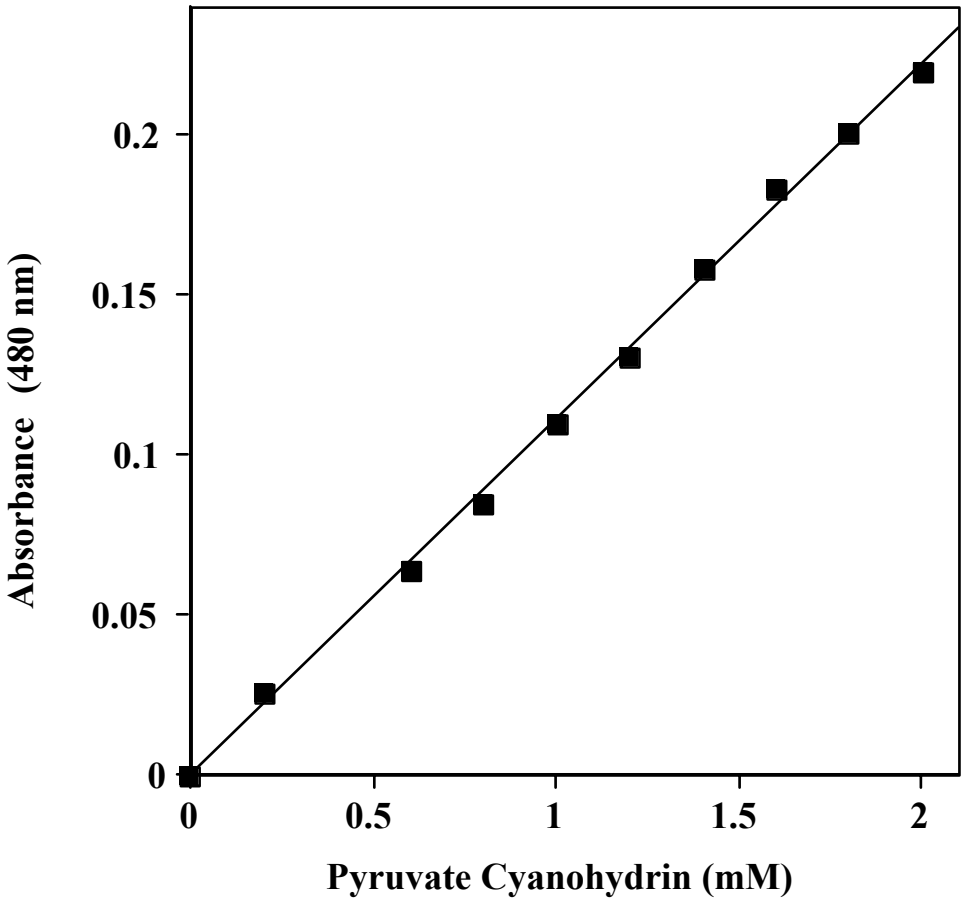
LKB Spectrophotometer, Perkin Elmer Lambda-2, Rainin HPLC system, AMINEX Ion Exclusion HPX-87H column, Rank Oxygen Electrode (Clark type), Liquid Scintillation counter Beckman LS 7000.

6. Chemicals.

KCN (97%) was obtained from Aldrich (Milwaukee, Wisconsin) and $K^{14}CN$ (50 mCi $mol^{-1}$, 1.9 GBq $mmol^{-1}$) was from DuPont. All other chemicals were of the highest purity commercially available. Cyanohydrin was routinely prepared in the laboratory by mixing solutions of 1M KCN and 100 mM keto acid in the ratio of 1:1 V/V. Reaction mixtures

were acidified immediately with 100 µl of 5 N HCl and incubated on ice for 20 minutes. This was followed by its overnight incubation in the refrigerator. Excess volatile cyanide was removed by purging the reaction mixture with nitrogen or by evacuation. It was found that the cyanohydrin preparations remained stable at $4^{o}$C under acidic conditions for 2-4 months with the relative concentrations checked periodically by HPLC. The pH of cyanohydrin solutions was adjusted to 7.0 just prior to its addition to enzymatic reaction mixtures to minimize interference from free cyanide.

Figure 1. Standard curve for pyruvate cyanohydrin determination by the alkaline picric acid method (Fry and Millar, 1972).


Absorbance (480 nm)
0.2
0.15
0.1
0.05
0
0
0.5
1
1.5
2
Pyruvate Cyanohydrin (mM)

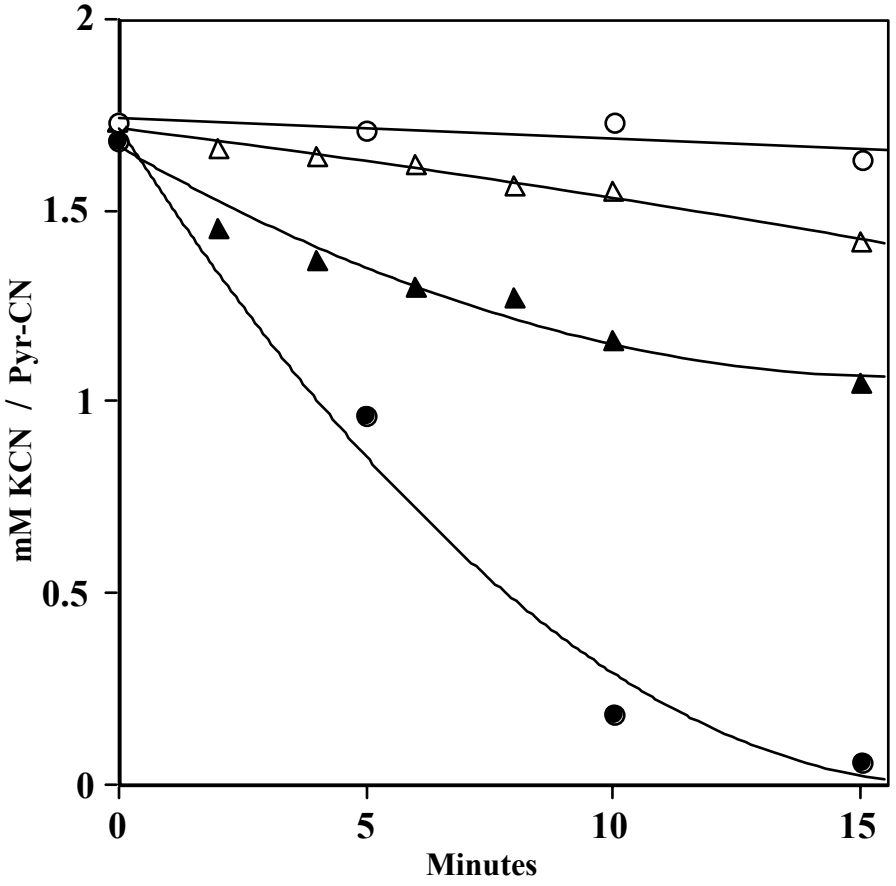
# CHAPTER III

# RESULTS

## PART I. Free cyanide is the true substrate for cell free cyanide oxidation activity.

1. Kinetics of cyanohydrin decomposition. Earlier studies by Kunz et al., (1998) showed that utilization of Pyr-CN and Kg-CN as sole nitrogen sources by *P. fluorescens* NCIMB 11764 required the induction of CNO, however, it was not established whether these compounds are attacked directly by the enzyme or metabolized by some other means. The involvement of CNO in cyanohydrin decomposition was therefore further investigated by comparing the kinetics of substrate disappearance with that of free cyanide (KCN). Figure 2 shows that Pyr-CN disappeared from reaction mixtures containing cell extracts induced for CNO much slower than did KCN. In addition, the rate of KCN disappearance was significantly higher than that observed for uninduced extracts demonstrating that CNO and cyanide oxidation are inducible (Kunz et al., 1992; Kunz et al., 1994). By comparison, extracts induced for CNO showed only a moderate stimulation in the rate of Pyr-CN disappearance over that observed with uninduced extracts (Fig. 2). The possibility that CNO was not directly involved in Pyr-CN decomposition was therefore considered. Subsequent experiments which showed that

Figure 2. Time course of pyruvate cyanohydrin (Pyr-CN) and free cyanide consumption by cell extracts of *P. fluorescens* NCIMB 11764. Reaction mixtures (0.25 ml) in 50 mM Na-K phosphate buffer (pH 7.0) contained 0.2 ml of cell extract (16 mg $ml^{-1}$), 4 mM NADH and 2 mM KCN. The remaining Pyr-CN and cyanide concentrations were determined as described in the Materials and Methods. Symbols: ▲ and Δ represent the remaining Pyr-CN in reaction mixtures, and ● and ❍ the remaining free cyanide in reaction mixtures supplied cell extract induced and uninduced for cyanide oxidation activity, respectively.


2
1.5
1
0.5
0
mM KCN / Pyr-CN
0
5
10
15
Minutes

Pyr-CN disappeared from reaction mixtures even in the absence of enzyme, and that its disappearance was accompanied by the simultaneous appearance of pyruvate and free cyanide in a time-dependent manner indicating that this compound was unstable under the assay conditions which included phosphate buffer at pH 7.0 (Fig. 3). It was hypothesized therefore, that the moderate stimulation in Pyr-CN disappearance from reaction mixtures in the presence of enzyme over that observed with uninduced extracts might be caused by the consumption of free cyanide accumulated in reaction mixtures. To test this hypothesis, the accumulation of cyanide was monitored both in the presence and absence of extracts induced for CNO. Figure 4 shows that with uninduced extracts cyanide readily accumulated in a time-dependent manner similar to that observed in nonenzymatic controls, however, in the presence of CNO no free cyanide was detected. It was therefore concluded that the enzyme consumes free cyanide formed spontaneously from Pyr-CN. Additional experiments showed that Kg-CN like Pyr-CN also dissociated in phosphate buffer at pH 7.0, however, both Pyr-CN and Kg-CN were stable at pH $\leq$ 4.0 indicating a strong pH effect on their stabilities. These results are consistent with the chemical behavior expected for $\alpha$-hydroxynitriles (cyanohydrins) in aqueous solution (Svirbely and Roth, 1953; Selmer et al., 1987; Majak et al., 1990), the dissociation of which is known to be favored at neutral to alkaline pH. However, this behavior has not generally been demonstrated for $\alpha$-hydroxynitriles containing the carboxy group such as Pyr-CN and Kg-CN. Thus, this work now shows that Pyr-CN and Kg-CN display similar chemical properties to other cyanohydrins in aqueous solution and exist in equilibrium with free cyanide and the respective $\alpha$-keto acid as shown in equation 5.

Figure 3. Time-course of spontaneous (■) pyruvate cyanohydrin (Pyr-CN) decomposition in Na-K phosphate buffer (pH 7.0). Reaction mixtures (0.25 ml) contained 2mM Pyr-CN and 4 mM NADH and at the times indicated samples were withdrawn and analyzed for the remaining Pyr-CN, free cyanide (●)and pyruvate (✧).

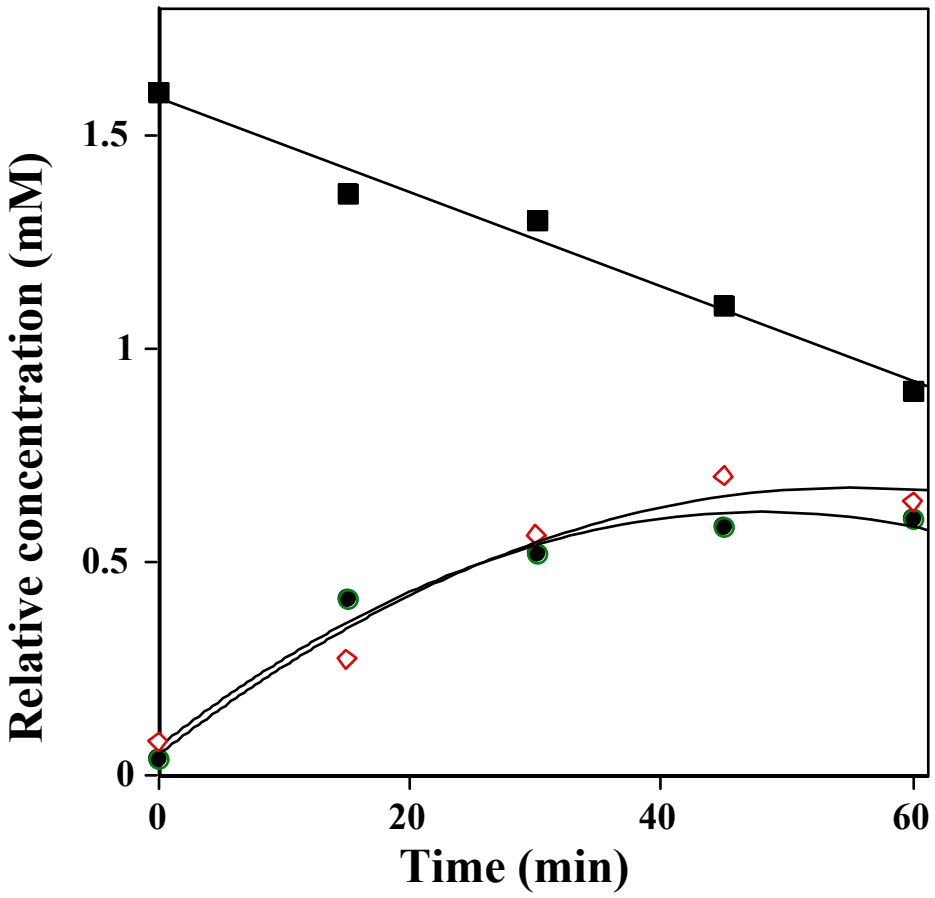
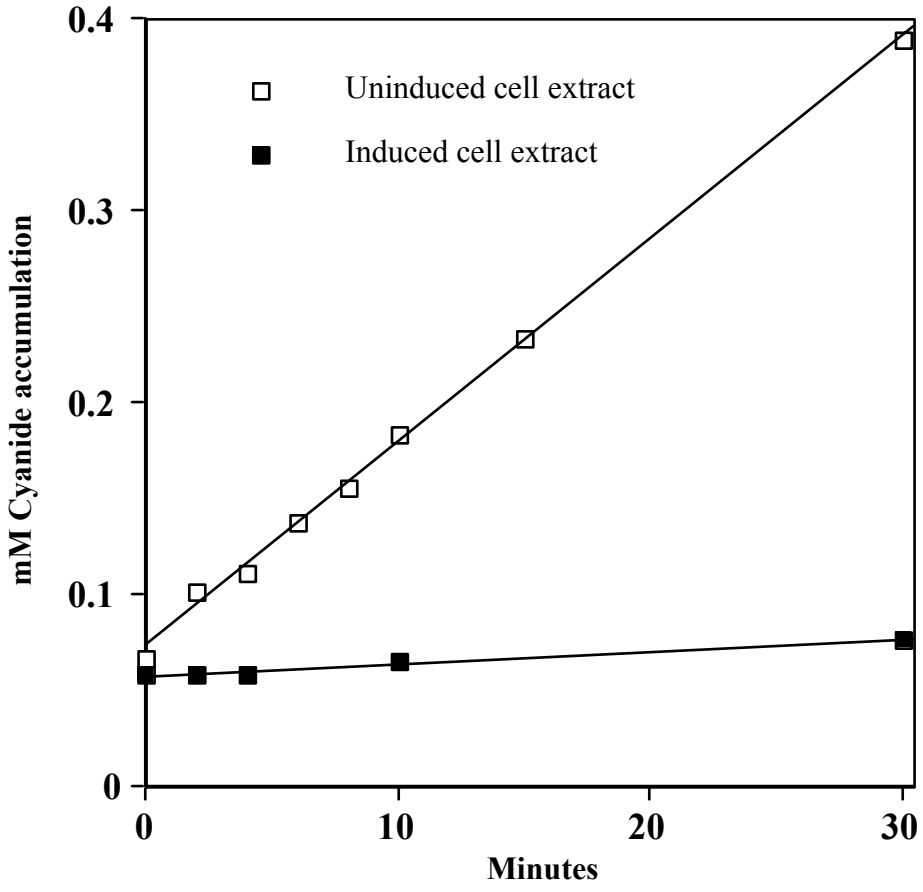
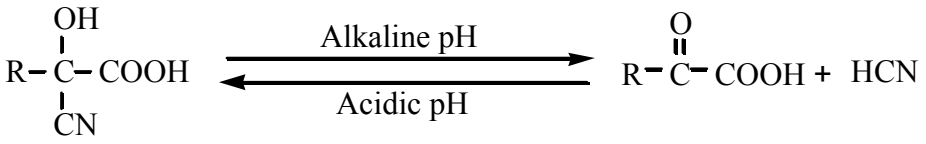

Relative concentration (mM)
1.5
1
0.5
0
0
20
40
60
Time (min)

Figure 4. Effect of CNO induction on the accumulation of cyanide from Pyr-CN. Reaction mixtures contained 2 mM Pyr-CN, 4 mM NADH, and 200 μl of cell extract (16 mg $ml^{-1}$ protein) induced (■) or uninduced (❑) for CNO. At various times, 10 μl samples were withdrawn and the appearance of cyanide determined as described in the Materials and Methods.


0.4
0.3
0.2
0.1
0
mM Cyanide accumulation
Uninduced cell extract
Induced cell extract
0
10
20
30
Minutes

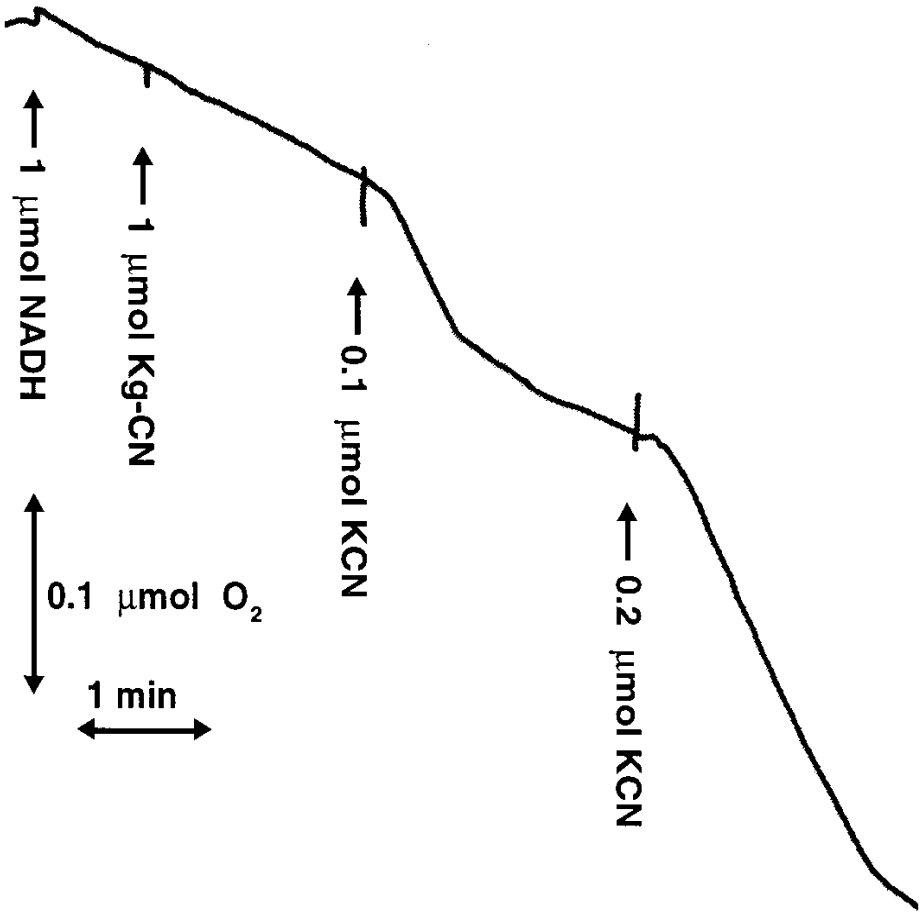
Equation 5




(R= $CH_3$ or HOOC-$CH_2$-$CH_2$)

2. Stimulation of oxygen uptake by cyanohydrin and free cyanide species. In order to further establish that CNO is incapable of attacking Pyr-CN and Kg-CN directly, enzyme activity measured as oxygen uptake towards these substrates was tested with an oxygen electrode. For these experiments special steps were taken to minimize the amount of free cyanide present in cyanohydrin solutions in order to avoid confusion over which chemical species (cyanide or cyanohydrin) was actually being oxidized. Figure 5 shows that Kg-CN had no effect on $O_2$ uptake and similar results were also observed for Pyr-CN. However, KCN caused an immediate stimulation in $O_2$ uptake with the amount of $O_2$ consumed equivalent to the amount of cyanide provided. Analogous experiments in which cyanide-free solutions of Pyr-CN and Kg-CN were tested for their ability to stimulate NADH consumption gave similar results (data not shown). Thus it was concluded that free cyanide is the substrate for CNO.

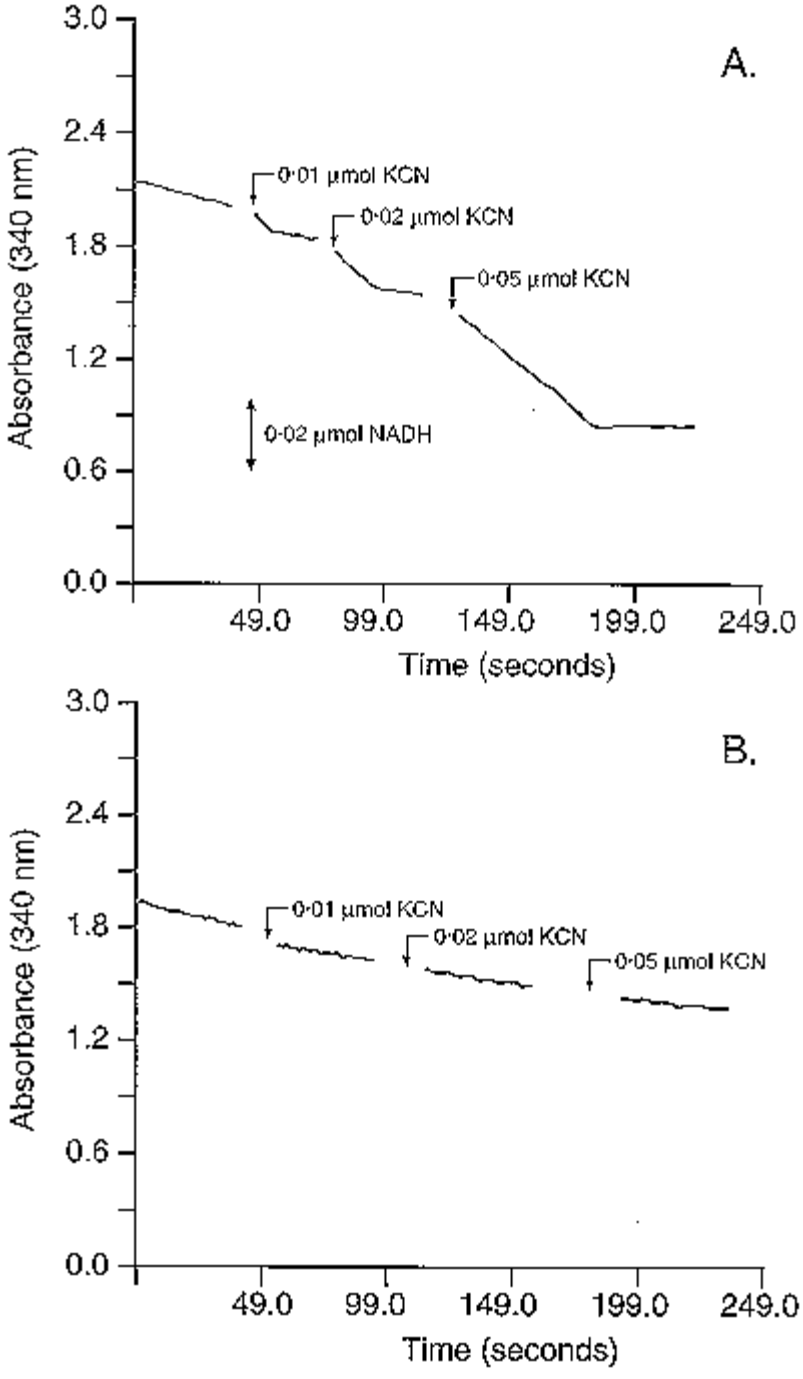
Figure 5. Oxygen uptake by cell extracts of *P. fluorescens* NCIMB 11764 induced for CNO towards ketoglutarate cyanohydrin (Kg-CN) and free cyanide (KCN). Reaction mixtures (1ml) in 50 mM Na-K phosphate buffer (pH 7.0) contained 2.4 mg protein and at the times indicated by the arrows, substrates were added in the amounts shown. Kg-CN (ketoglutarate cyanohydrin) was adjusted from pH 4.0 to pH 7.0 immediately before addition to reduce the interference from free cyanide as described in the Materials and Methods.


1 µmol NADH
1 µmol Kg-CN
0.1 µmol KCN
0.2 µmol KCN
0.1 µmol O2
1 min

**PART II. Cyanide oxidation involves a complex mechanism.**

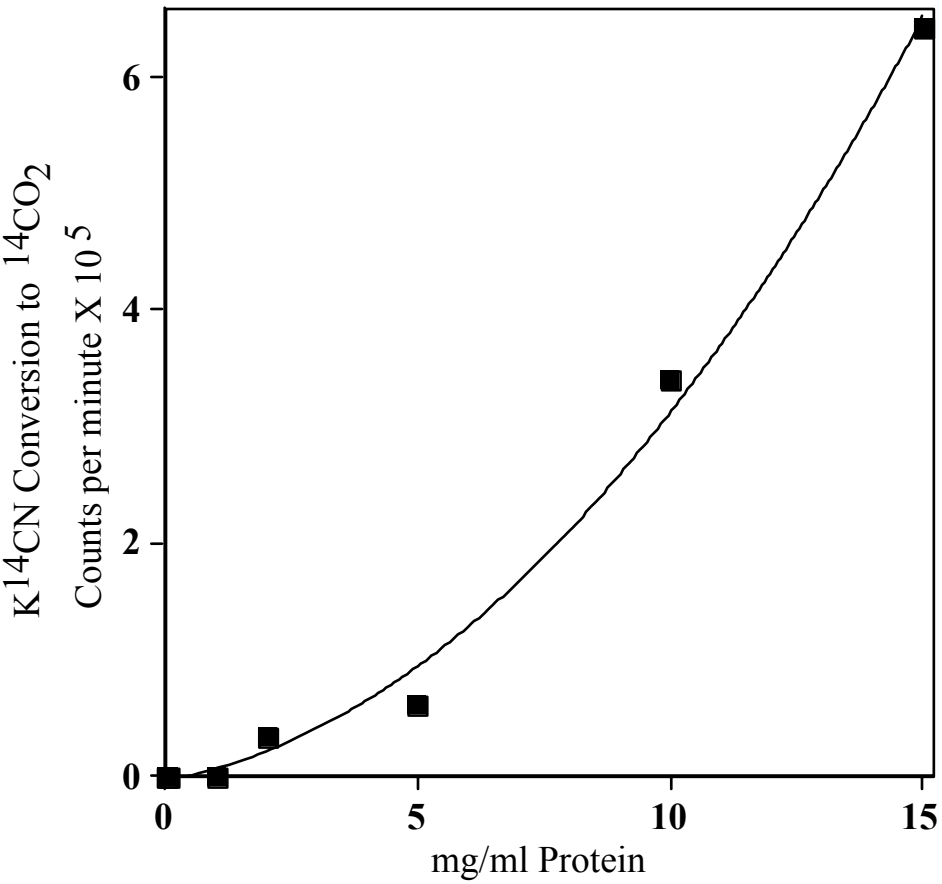
1. Development of improved assays for cyanide oxidation activity. The standard method for quantifying cyanide oxidation activity routinely used in our laboratory has been based on the measurement of substrate disappearance rates (Fig. 2). However, there are drawbacks with this approach given that samples must physically be removed during incubation and the remaining cyanide concentration determined in a somewhat laborious colorimetric assay. The possibility that some cyanide loss due to volatilization can occur during sampling is also a concern. Additional methods for reliably quantifying cyanide oxidation activity in cell extracts were therefore explored. Since both $O_2$ and NADH are required by CNO the consumption of each was tested as an alternative approach for determining enzyme activity. Figure 5, shows that cyanide was capable of stimulating $O_2$ uptake in a stoichiometric manner; as already discussed, these methods were used to establish that free cyanide (and not cyanohydrin) is the substrate for CNO. Figure 6 shows that similar results were obtained when NADH oxidation was measured as a function of cyanide concentration. In either case 1 mole each of $O_2$ and NADH were consumed per mole cyanide provided. These results agree with earlier studies on the stoichiometry of cyanide oxidation by Kunz and coworkers (Kunz et al., 1994; Wang, 1995) and are consistent with that expected for an oxygenase reaction in which $O_2$ and NADH are each consumed during substrate degradation. Figure 6B shows that no NADH oxidation activity could be detected in cell extracts prepared from uninduced cells thus further establishing the inducible nature of the CNO enzyme.

Figure 6. Effect of cyanide on NADH oxidation by cell extracts induced (A) and uninduced (B) for cyanide oxidation activity. Reaction mixtures (0.5 ml) in 50 mM Na-K phosphate buffer (pH 7.0) contained 0.4 mM NADH, and at the times indicated KCN was added in the amounts shown.


A.
3.0
2.4
1.8
1.2
0.6
0.0
Absorbance (340 nm)
0·01 µmol KCN
0·02 µmol KCN
0·05 µmol KCN
0·02 µmol NADH
49.0
99.0
149.0
199.0
249.0
Time (seconds)
B.
3.0
2.4
1.8
1.2
0.6
0.0
Absorbance (340 nm)
0·01 µmol KCN
0·02 µmol KCN
0·05 µmol KCN
49.0
99.0
149.0
199.0
249.0
Time (seconds)

2. Kinetics of cyanide oxidation by cell free extracts. Having established that the consumption of $O_2$ and NADH can be used as sensitive methods for quantifying cyanide oxidation activity, these methods were used to investigate the kinetics of enzymatic conversion. Initial experiments aimed at determining the effect of protein concentration on enzyme activity gave a nonlinear relationship between the rate of $O_2$ (or NADH) consumption and the amount of protein supplied (when measured at a standard cyanide concentration of 2 mM and 1-5 mg $ml^{-1}$ protein)(data not shown). Related experiments in which the production of radiolabelled $^{14}CO_2$ from $K^{14}CN$ (or the disappearance of cyanide) was measured as function of protein concentration gave similar results (Fig.7) These observations find analogy with biochemical conversions catalyzed by other oxygenase enzymes for which the nonlinearity between enzyme activity and protein has generally been attributed to the multicomponent nature of such enzymes (Gibson D. T., 1988; Mason and Cammack, 1992; An et al., 1994). Alternatively, other explanations may account for the results observed, but regardless, a complex mechanism of enzymatic conversion is implied. The requirement for large amounts of protein (10 mg $ml^{-1}$) for optimal enzymatic conversion further implies a complex enzymatic mechanism. Studies in which enzyme activity assayed by $O_2$ consumption was measured as a function of substrate concentration revealed activity over a broad substrate concentration range (0.01-100 mM). However, regardless of the cyanide concentration provided, no difference in the rate of $O_2$ consumption could be observed. Analogous results were also obtained when NADH oxidation as a function of cyanide concentration was determined.

Figure 7. Conversion of radioactive $K^{14}CN$ to $^{14}CO_2$ as a function of protein concentration by cell extracts induced for cyanide oxidation activity. Separate reactions supplied KCN (2 mM), $K^{14}CN$ (1μCi) and 4 mM NADH were incubated for 10 minutes at which time reactions were terminated and radioactivity as volatile $^{14}CO_2$ (plus $H^{14}CO_3^-$) determined.

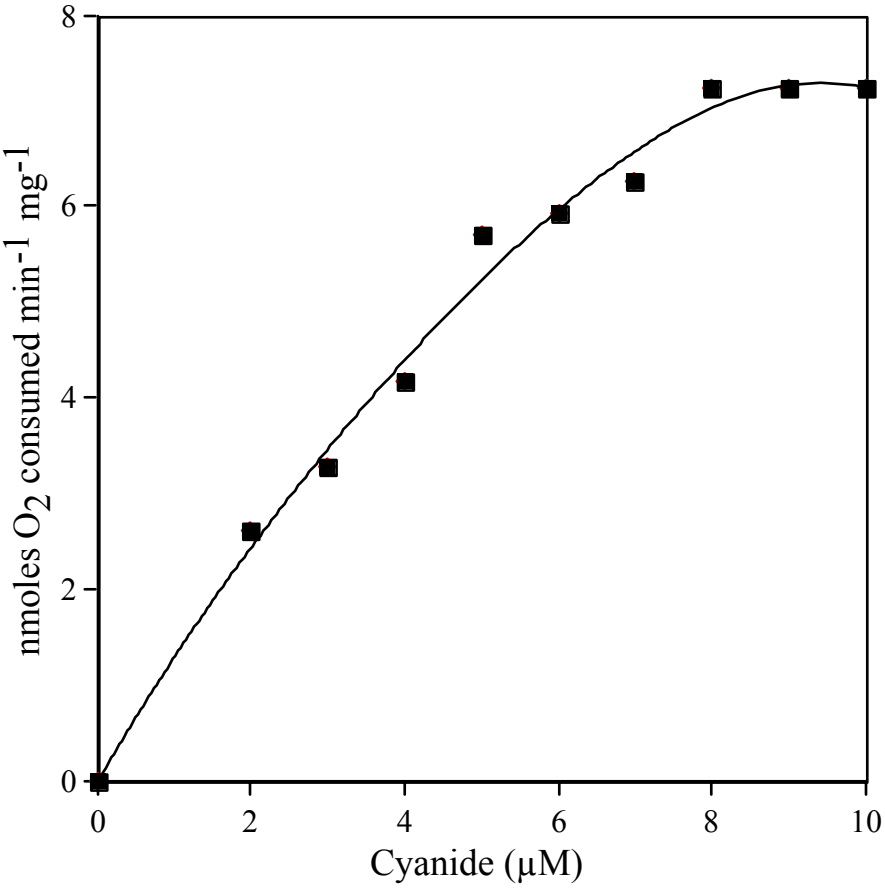

K14CN Conversion to 14CO2
Counts per minute X 10 5
0
2
4
6
0
5
10
15
mg/ml Protein

Two possible explanations were thought of to account for these results. First, either the rate of $O_2$ (or NADH) consumption was independent of substrate concentration, or the amount of substrate needed to saturate the enzyme was below 10 μM. In order to distinguish between these possibilities $O_2$ uptake was measured at a KCN concentration ranging from 1 and 10 μM, and as illustrated in Figure 8, under these conditions $O_2$ uptake increased in a substrate-concentration dependent manner. Maximal $O_2$ uptake was observed at a KCN concentration of 8 μM with the corresponding half saturation constant ($Km_{app}$) was estimated at 4 $\pm$ 0.5 μM. The specific activity for CNO using this approach was determined to be ~ 7 nmol $O_2$ consumed $min^{-1}$ $mg^{-1}$ protein. This agrees with values reported previously from substrate consumption assays (Kunz et al., 1994), however, the apparent Km estimated for the enzyme towards cyanide is 300 times lower than that previously reported (1.2 mM). The explanation for this is believed to be due to the greater sensitivity of $O_2$ uptake measurement as opposed to substrate consumption (which as indicated already has technical drawbacks). Thus, the apparent affinity of CNO for cyanide was shown to be far greater than that earlier recognized thus providing a reasonable explanation for how cyanide may be oxidized at low physiological concentrations encountered during growth. Curiously, the enzyme also displayed a remarkable tolerance to cyanide, since activity at concentrations as high as 100 mM was detected. However, as described below a separate nonenzymatic process was shown to be responsible for this effect.

3. Enzymatic and nonenzymatic processes are involved in cyanide-dependent $O_2$ uptake.

Results showing that the half-saturation constant for cyanide (4 μM) when

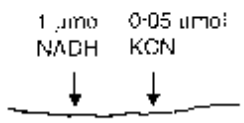
Figure 8. $O_2$-uptake as a function of KCN concentration by cell extracts induced for CNO. Reaction mixtures (0.5 ml) in Na-K phosphate buffer (pH 7.0) contained 2.0 mg protein, 4mM NADH and KCN at the concentration shown. Values shown represent the means of three separate determinations.


8
6
4
2
0
0
2
4
6
8
10
nmoles O2 consumed min-1 mg-1
Cyanide (µM)

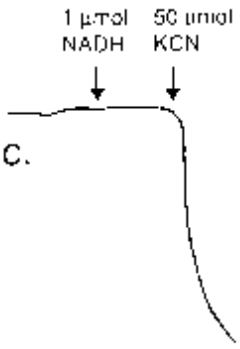
measured by $O_2$ consumption was almost two orders of magnitude lower than the maximum concentration of cyanide (100 mM) at which activity was detected suggested that two separate $O_2$-dependent processes might be operative at low (10-100 μM) and high (10-100 mM) cyanide concentrations. Findings published in the mid-eighties by I. Fridovich and coworkers (Robertson et al., 1981; Mashino and Fridovich, 1987) which showed that cyanide can chemically catalyze the autooxidation of certain chemical species led us to hypothesize that a similar process might account for $O_2$ uptake at high cyanide concentrations. To determine this, boiled extracts (95$^{o}$C for 10 min) were similarly tested for the ability to stimulate $O_2$ consumption in the presence of cyanide. Figure 9b shows that the addition of cyanide at low concentration to unboiled extracts resulted in an immediate stimulation in $O_2$ uptake, however, no such activity was observed with boiled extracts (Fig. 9a). These results were interpreted as indicating that CNO is destroyed by boiling. However, at high concentration (100 mM) rapid $O_2$ uptake was observed in both boiled and unboiled extracts (Fig. 9c & d). Based on these observations it was concluded that a heat stable factor present in boiled extracts was responsible for the observed cyanide-related oxidation activity. The nature of this factor was not further investigated, however, it is hypothesized that a flavin species whose autooxidation is cyanide-mediated may be involved. That an autooxidation mechanism is operative is further suggested by results showing that the rate and extent of $O_2$ uptake was dependent both on the amount of boiled extract and cyanide supplied (data not shown).

Figure 9. Comparison of oxygen uptake by boiled and unboiled extracts at different cyanide concentrations. Reaction mixtures in 0.5 ml Na-K buffer (pH 7.0), contained either boiled (1mg/ml protein)(a and c) or unboiled (4mg/ml protein) (b and d) cell extract, 1μmol NADH and KCN in the amount indicated.

**BOILED**

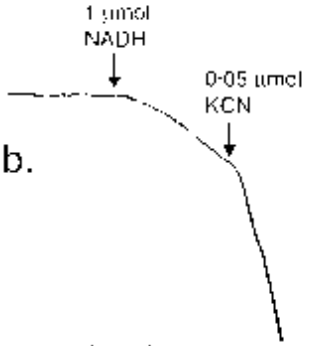



a.

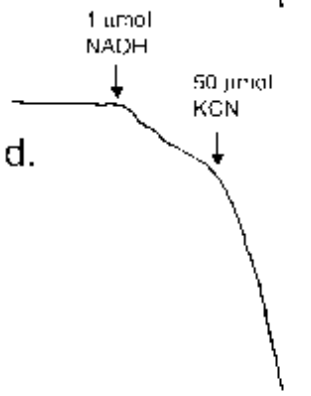



c.

**UNBOILED**




b.

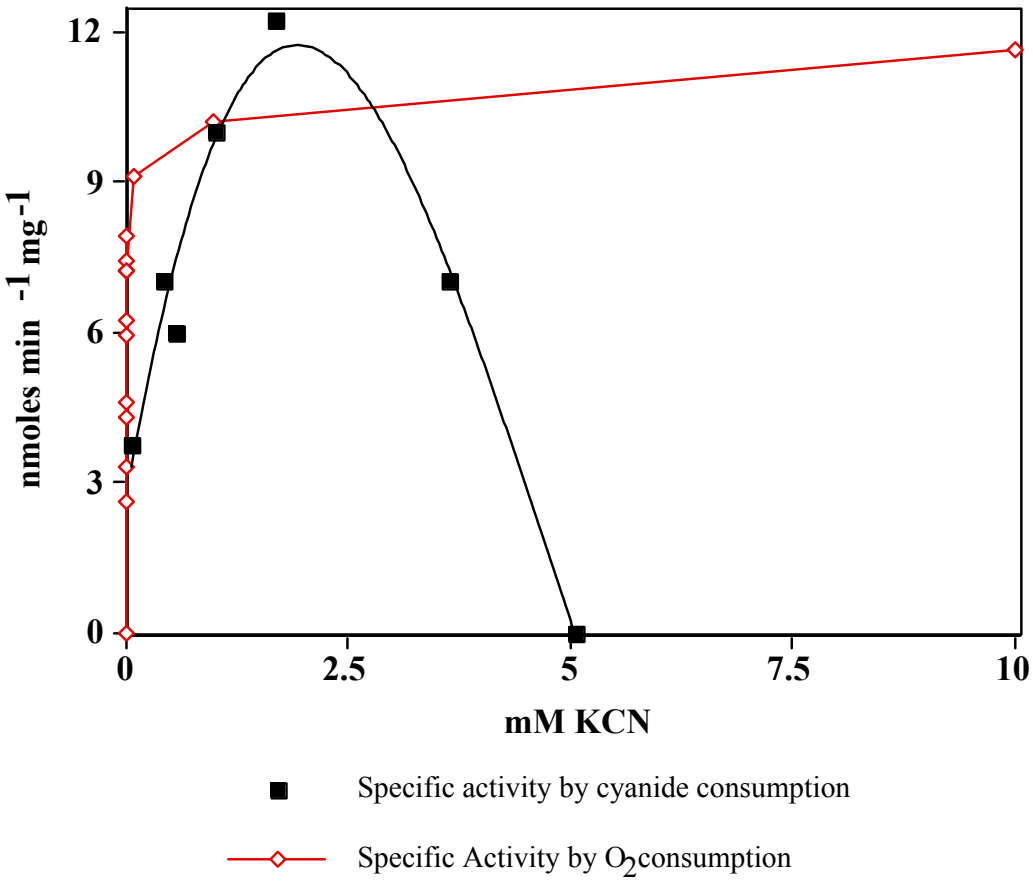



d.

4. CNO activity is required for cyanide turnover.

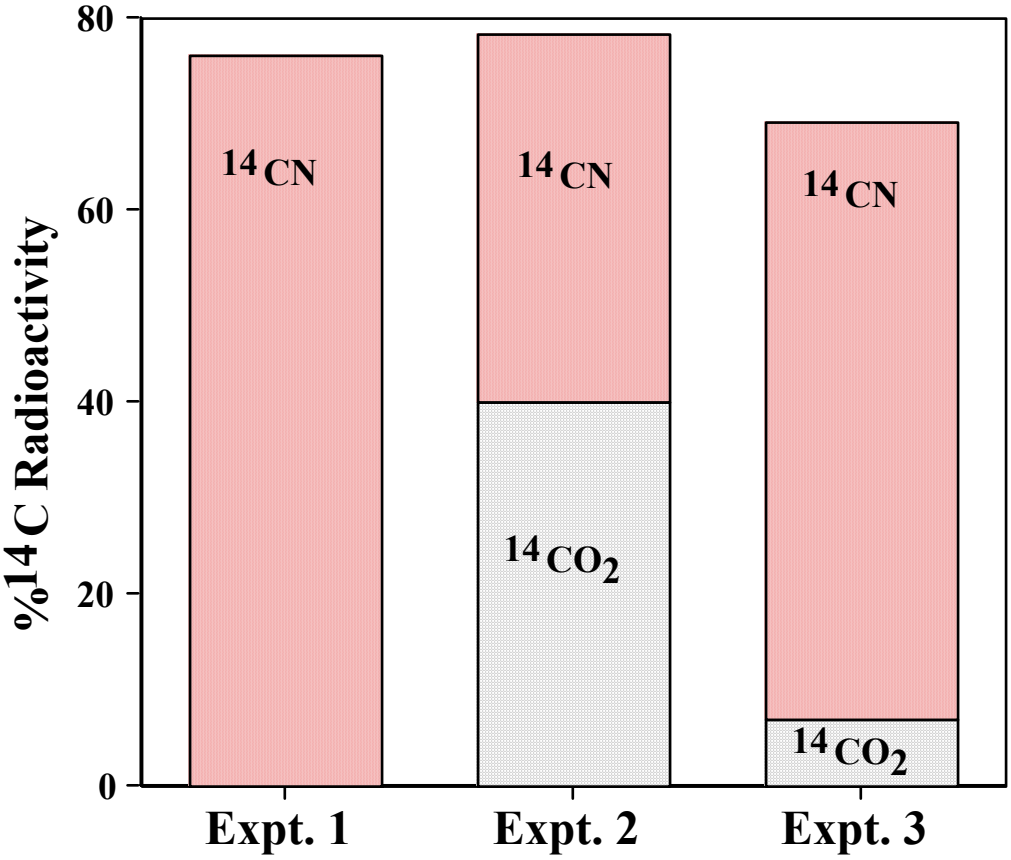
In order to show that cyanide turnover was linked to $O_2$ consumption and that this process was CNO dependent, the relationship between the two was investigated at various substrate concentrations. Figure 10 shows that the addition of cyanide at micromolar concentrations to extracts induced for CNO caused an immediate burst in $O_2$ uptake. These observations are similar to those already described (see Fig. 8) from which an apparent half-saturation constant for cyanide as determined by $O_2$ uptake was estimated at 4 μM. Figure 10 further shows no significant difference in the rate of $O_2$ consumption by cell extracts when the concentration of KCN was varied between 10 μM or 10 mM (or up to 100 mM). These results are consistent with our interpretation that two separate cyanide-dependent processes are involved in $O_2$ uptake, one being enzymatic (CNO) in nature (and operative at low cyanide [1-10 μM]), and the other being nonenzymatic in nature (and operative at high cyanide [0.01-100 mM]). When activity based on substrate consumption was compared with that of $O_2$ consumption a completely different pattern was observed. First, the maximal rate of cyanide consumption (10-12 nmol $min^{-1}$ $mg^{-1}$) was found to be almost identical to that achieved by $O_2$ consumption; the optimal cyanide concentration required, however, was dramatically different (i.e., 10 μM for $O_2$ uptake versus 2 mM for CN consumption). We again interpret this large discrepancy as being due to differences in sensitivities of the two assays (it is not possible to measure cyanide accurately in the micromolar range during enzymatic conversion because of complications in removal of samples for colorimetric cyanide determination). A second major observation was that cyanide

Figure 10. Cyanide oxidation activity in cell extracts as a function of cyanide concentration measured either as $O_2$ (✧) or cyanide consumption (■). Reaction mixtures (0.5 ml) in 50 mM Na-K phosphate buffer (pH 7.0) contained 2 mg protein (4 mg $ml^{-1}$), and NADH at two times the indicated KCN concentration.


12
9
6
3
0
nmoles min -1 mg-1
0
2.5
5
7.5
10
mM KCN
Specific activity by cyanide consumption
Specific Activity by O2consumption

consumption, while increasing as a function of substrate concentration, eventually declined and was completely inhibited at 5 mM. These results contrasted sharply with parallel $O_2$ uptake assays and implied that cyanide consumption was subject to cyanide inhibition. These results were most interesting because for the first time, evidence that CNO can also be inhibited by cyanide was obtained. They also provided support for the hypothesis that the optimal substrate concentration for CNO activity is probably in the micromolar range. It is important to note that the protein concentration in these experiments was relatively low (4 mg $ml^{-1}$) in comparison with that known to support maximal cyanide conversion (see Fig 7). In separate experiments (data not shown) where the protein concentration was increased to 10 mg $ml^{-1}$, tolerance to cyanide simultaneously increased (e.g., 100% inhibition occurring at 15 mM KCN) suggesting that CNO can somehow be protected from the inhibitory effects of cyanide at higher protein concentrations. This may explain, in part, the sigmoidal relationship between activity and protein concentration as described earlier (Results section: Kinetics of cyanide oxidation by cell free extracts, Fig 7). To further confirm that cyanide consumption at low substrate concentrations was correlated with its enzymatic turnover extracts were incubated with radioactive $K^{14}CN$ and its conversion to $^{14}CO_2$ determined (Fig. 11). These data illustrate that $K^{14}CN$ supplied at 80 μM was converted in almost 50% yield to $^{14}CO_2$ by extracts induced for CNO. In contrast, boiled extracts incubated similarly converted less than 1% of the available $K^{14}CN$ to $^{14}CO_2$, thus providing strong evidence that cyanide turnover is indeed enzyme-dependent. Figure 11 (Expt. 3) further shows that when extracts were incubated with $K^{14}CN$ in the presence of a large excess of

Figure. 11. Cyanide ($K^{14}CN$) turnover to $^{14}CO_2$ by unboiled (Expts 2 and 3) and boiled (Expt 1) cell extracts of *Pseudomonas fluorescens* NCIMB 11764. For experiments 1 and 2, reaction mixtures in 0.25 ml Na-K phosphate buffer (pH 7.0) contained 1μCi $K^{14}CN$ (80 μM), 4 mM NADH and 4 mg $ml^{-1}$ cell extract protein. Reactions were allowed to incubate for 5 minutes at 30°C and radioactivity as volatile $^{14}CO_2$ (plus $H^{14}CO_3^-$) determined. The reaction conditions for Experiment 3 were identical except that 20 mM nonradioactive KCN and 40 mM NADH were supplied and the reaction was allowed to proceed for 60 minutes before $^{14}CO_2$ was determined.


80
60
40
20
0
%14C Radioactivity
14CN
14CN
14CN
14CO2
14CO2
Expt. 1
Expt. 2
Expt. 3

unlabelled KCN (high cyanide [20 mM]), only 10% of the substrate was converted to $CO_2$, presumably, caused by the inhibitory effect of cyanide on CNO.

# CHAPTER IV

# DISCUSSION

Pyruvate and α-ketoglutarate accumulate in culture supernatants when *P. fluorescens* 11764 is grown under nitrogen limiting conditions (Chen and Kunz, 1997; Kunz et al., 1998). These compounds react spontaneously with cyanide to give less toxic cyanohydrins, which are further metabolized. Earlier studies on the metabolism of the cyanohydrin substrates by *P. fluorescens* NCIMB 11764 showed that (i) the $^{14}C$-labelled cyanohydrins were converted by cell extracts to $^{14}CO_2$ (and $NH_3$), (ii) conversion required oxygen and NADH, and (iii) a mutant strain defective in the production of CNO was unable to effectively metabolize the cyanohydrins as nitrogen sources (Kunz et al., 1998). These studies implied that CNO was somehow involved in cyanohydrin metabolism, but details of its involvement were not further pursued. In this study, the involvement of CNO in cyanohydrin utilization was further investigated. Results obtained now show that CNO is only indirectly involved in the further breakdown of the cyanohydrins as substrates. Both Pyr-CN and Kg-CN were found to be unstable at neutral pH and spontaneously dissociated into free cyanide and the respective keto acids (equation 5). Since free cyanide could not be detected in reaction mixtures containing CNO, it can be concluded that the enzyme consumes the free cyanide formed when the cyanohydrins spontaneously dissociate. Results demonstrating that the rate of Pyr-CN disappearance

was only marginally stimulated by extracts above that occurring nonenzymatically are further consistent with an indirect role for the enzyme in cyanohydrin decomposition. Further evidence for this was provided by oxygen uptake experiments, which showed that neither Pyr-CN nor Kg-CN were capable of stimulating $O_2$ consumption. In contrast, free cyanide was readily oxidized with an established stoichiometry of 1:1:1 for $O_2$, NADH and cyanide, respectively. Thus, despite earlier studies (Kunz et al., 1998) which demonstrated that cyanide detoxification by chemical conversion to the cyanohydrins is an important first step in its assimilation as a growth substrate, this study showed that free cyanide remains the substrate for enzymatic attack. In addition, since cyanide exists predominantly in its protonated form at pH 7.0, given a pKa of 9.3 (Fuller, 1984), it may be inferred that HCN and not $CN^-$ interacts directly with the enzyme.

Studies on the oxidation of cyanide by cell-free extracts as performed in Part II of this thesis revealed important new findings on the kinetics and conditions under which cyanide is oxidized. For example, a nonlinear relationship between enzyme activity and protein concentration was consistently observed regardless of the method used to measure enzyme activity (i.e., substrate consumption, $O_2$ or NADH consumption, or measuring the rate of $K^{14}CN$ conversion to $^{14}CO_2$ [Fig 7]). These results imply that cyanide oxidation probably involves a complex mechanism. Part of this complexity is likely due to the properties of CNO itself, which by analogy with other oxygenase enzymes, probably exists as a multicomponent enzyme. The requirement for large amounts of protein for activity as earlier also reported (Kunz et al., 1994) can possibly be

explained by the multicomponent nature of the enzyme and the dependence on protein aggregation for catalytic activity.

Additional studies on the effect of substrate concentration on enzymatic activity were also performed, but for this purpose it was necessary to develop new assays given the inherent difficulties associated with measuring substrate consumption. The measurement of cyanide-dependent $O_2$ and NADH consumption proved effective for this purpose, since both are utilized directly by putative CNO. When tested as a function of cyanide concentration ranging from 0.1 to 100 mM no difference in the initial rate of either $O_2$ or NADH consumption by cells extracts were observed. It was subsequently determined that this was because the amount of cyanide required to achieve saturating levels of activity was much lower than 0.1 mM. Maximal activity (10-12 nmol $O_2$ consumed $min^{-1}$ $mg^{-1}$) was eventually demonstrated at a KCN concentration of 8 μM; the deduced half-saturation constant for cyanide ($Km_{app}$) is therefore 4.0 $\pm$ 0.5 μM. This value is approximately 300 times lower than that previously estimated from substrate consumption rates (Kunz et al., 1994) and suggests strongly, that the affinity of CNO for cyanide is quite high. These observations have important implications for understanding the physiology of cyanide utilization. For example, previous studies from this laboratory demonstrated that the detoxification of cyanide by complexation to α-keto acids is an important prerequisite for its assimilation as a growth substrate. (Kunz et al., 1998). Studies described in Part I of this thesis further showed that the corresponding cyanohydrins, formed when cyanide reacts with α-keto acids accumulated by cells in the medium, are not directly metabolized but give rise to free cyanide which is then oxidized

by CNO. It may be inferred therefore, that following cyanide complexation in the extracellular fluid, the cyanohydrins enter the cell where they release cyanide at low concentrations. Preliminary experiments have shown that intact cells readily metabolize Pyr-CN and Kg-CN and previous studies also showed that these compounds can in fact support growth (Kunz et al., 1998; Parab and Kunz, 2000). These findings provide good evidence that these compounds are readily taken up by cells. The apparent high affinity of CNO for free cyanide can therefore be viewed as an advantage since once cyanide is released intracellularly, it presumably is rapidly attacked by CNO thereby reducing its effective intracellular concentration and at the same time providing ammonia for physiological growth.

Research findings on the metabolism of cyanide-containing cyanohydrin substrates by *P. fluorescens* NCIMB 11764 find important parallels with related studies on the utilization of the metal-cyano complex, tetracyanonickelate (II)($[Ni(CN_4)]^{2-}$)(TCN) as a sole nitrogen source. Utilization of TCN by *P. fluorescens* NCIMB 11764 was shown also to be CNO-dependent (Rollinson, et al., 1987; Kunz et al., 1990) and comparative studies with other TCN-utilizing bacteria isolated in our laboratory have shown that the ability to grow on TCN is consistently paralleled by the ability to grow on KCN (Silva-Avalos et al., 1990). Further studies indicate that the degradation of TCN also proceeds via the release of free cyanide and not by direct enzymatic attack on the metal complex (Silva-Avalos et al., 1990). Although TCN is presumably much more stable than the cyanohydrins (the stability constant for TCN is $10^{-12}$ [Dawson et al., 1969]), CNO-catalyzed consumption of even small amounts of free cyanide likely to arise by

dissociation of TCN may help explain how this compound is also capable of supporting growth. TCN, like Pyr-CN and Kg-CN is also significantly less toxic than free cyanide (MIC = 50 mM ver 0.3 mM for KCN)(Silva-Avalos, et al., 1990; Pan, 1998). Thus, a model for the utilization of cyanide as a growth substrate can be proposed (Scheme 2) in


**Scheme 2. Model for indirect catalysis of cyanide complex decomposition. Lig, refers to ligand as represented either by the pyruvate or ketoglutarate in cyanohydrins or $Ni^{2+}$ in TCN. CNO, cyanide oxygenase**

which cyanide is acquired from nontoxic complexes such as Pyr-CN, Kg-CN or TCN by enzymatic assimilation of free cyanide, which arises from the complexes at low sublethal levels. Such complexes presumably enter the cell (or dissociate at the cell surface) making cyanide available for enzymatic attack. By this means, the potent toxicity of cyanide can presumably be avoided while the nutritional benefit of cyanide as a nitrogen source can still be taken advantage of.

While CNO from *P. fluorescens* NCIMB 11764 presumably attacks cyanide at micromolar concentrations, it was surprising to find that cyanide could stimulate the rate of $O_2$ uptake at concentrations as high as 100 mM; at these concentrations it might be expected that the enzyme would be strongly inhibited. Experiments which demonstrated that extracts with no CNO activity as a result of boiling still were able to consume $O_2$ suggested strongly that a separate nonenzymatic process was responsible for this effect. Since other studies have demonstrated that cyanide can chemically catalyze the

autooxidation of certain chemical species such as α-hydroxyaldehydes (Robertson, et al., 1981; Mashino and Fridovich, 1987) and flavanoids (Hodnick, et al., 1986) we hypothesize that a related mechanism is responsible for the cyanide-dependent $O_2$ uptake observed particularly at high cyanide concentrations. The responsible species could be a heat stable flavin species, since oxygen uptake was repeatedly shown to be stimulated by NADH. Additional experiments not included in this thesis (Parab and Kunz, 2000) demonstrated that the oxidation of the unknown autooxidizable species in extracts could be coupled to the reduction of cytochrome c. Since cytochrome c participates in one-electron transfer reactions only, it may be deduced that a mechanism involving single electron removal from the responsible species is involved. This agrees with other cyanide-catalyzed autooxidation mechanisms for which dioxygen or cytochrome c have been reported as appropriate electron acceptors (Robertston et al., 1983; Mashino and Fridovich, 1987).

Earlier studies (Kunz et al., 1992; Kunz et al., 1994; Wang C. S., 1996; Kunz et al., 1998) provided evidence that utilization of cyanide as the sole nitrogen source requires the induction of a unique cyanide monoxygenase. This conclusion was based on evidence which showed that (i) both $O_2$ and NADH were required, (ii) enzyme activity in cell extracts could not be detected in a mutant strain having lost the ability to grow, and (iii) isotopically molecular oxygen was incorporated during conversion. Although the enzyme remains to be purified, the results of this study have further confirmed the requirement of this putative enzyme for cyanide oxidation. Experiments demonstrating the lack of conversion of radiolabelled cyanide to $^{14}CO_2$ by boiled extracts provide strong evidence

that the enzyme is indeed required for substrate turnover (Fig. 11). In addition, the reaction stoichiometry between $O_2$, NADH and cyanide consumed (1:1:1) was further confirmed. Two additional discoveries made in this work provide yet further evidence of the importance of CNO in cyanide metabolism. First, the fact that cyanide was shown to be attacked at micromolar concentrations lends further support to the idea that CNO participates physiologically in cyanide utilization. Second, for the first time it was shown that paradoxically, while the enzyme oxidizes the substrate at low concentrations, it is also subject to inhibition (Figs. 10 and 11). These results can be interpreted as indicating that, like other oxygenases, CNO also exists as a metalloenzyme. The nonlinear kinetics between activity and enzyme concentration provides additional suggestive evidence of this since such kinetics are usually indicative of complex protein interactions typical of such multicomponent enzymes.

This study has demonstrated that enzymatic degradation of cyanide involves an apparent interaction between the small molecule cyanide, and a unique enzyme formed by *P. fluorescens* NCIMB 11764 cells. To further understand the molecular nature of this interaction the protein or proteins constituting CNO will have to be purified. In addition, whether additional enzymes besides CNO are also needed for complete cyanide oxidation to $CO_2$ and $NH_3$ remains to be determined. Further studies on the nature of these proteins in *P. fluorescens* NCIMB 11764 and the occurrence of possible related enzymes in other cyanide-utilizing bacteria should provide important clues about the unique physiology and biochemistry involved in the metabolism of toxic cyanide in nature.

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